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A Plant Homeodomain in Rag-2 that Binds Hypermethylated Lysine 4 of Histone H3 Is Necessary for Efficient Antigen-Receptor-Gene Rearrangement

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SUMMARY

V(D)J recombination is initiated by the recombination activating gene (RAG) proteins RAG-1 and RAG-2. The ability of antigen-receptor-gene segments to undergo V(D)J recombination is correlated with spatially- and temporally-restricted chromatin modifications. We have found that RAG-2 bound specifically to histone H3 and that this binding was absolutely dependent on dimethylation or trimethylation at lysine 4 (H3K4me2 or H3K4me3). The interaction required a noncanonical plant homeodomain (PHD) that had previously been described within the noncore region of RAG-2. Binding of the RAG-2 PHD finger to chromatin across the IgH D-J_H-C locus showed a strong correlation with the distribution of trimethylated histone H3 K4. Mutation of a conserved tryptophan residue in the RAG-2 PHD finger abolished binding to H3K4me3 and greatly impaired recombination of extrachromosomal and endogenous immunoglobulin gene segments. Together, these findings are consistent with the interpretation that recognition of hypermethylated histone H3 K4 promotes efficient V(D)J recombination in vivo.

INTRODUCTION

The antigen-receptor genes of lymphocytes are encoded in separate DNA segments that are brought together by variable-diversity joining [V(D)J] recombination (Fugmann et al., 2000a; Gellert, 2002). Recombination activating gene (RAG) proteins RAG-1 and RAG-2 initiate V(D)J recombination by cleaving participating gene segments at recombination signal sequences (RSSs), producing two signal ends, which terminate in double-stranded breaks, and two coding ends, which terminate in hairpin structures (Gellert, 2002). V(D)J recombination is directed toward specific antigen-receptor loci in an ordered fashion during lymphoid development. In the B lineage, for example, the immunoglobulin heavy chain (IgH) locus is rearranged before the light chain loci, and within the IgH locus, D-to-J_H joining preceeds V_H-to-DJ_H joining (Alt et al., 1984). This specificity might be enforced by epigenetic mechanisms that regulate access of antigen-receptor loci to RAG-1 and RAG-2 (Cobb et al., 2006). The ability of antigen-receptor-gene segments to undergo V(D)J recombination is correlated with spatially- and temporally-restricted chromatin modifications, including histone acetylation (Chowdhury and Sen, 2001; Chowdhury and Sen, 2003; Goldmit et al., 2005; Johnson et al., 2003; McMurry and Krangel, 2000) and methylation (Goldmit et al., 2005; Johnson et al., 2004; Morshead et al., 2003; Perkins et al., 2004).

Recombinationally active regions are typically marked by hyperacetylated histones H3 and H4, as well as lysine 4-methylated histone H3 (Chowdhury and Sen, 2001; Chowdhury and Sen, 2003; Goldmit et al., 2005; Johnson et al., 2003; Johnson et al., 2004; McMurry and Krangel, 2000; Morshead et al., 2003; Perkins et al., 2004). Peaks of histone H3, dimethylated at lysine 4 (H3K4me2), flank the D-J_H cluster in pro-B cells poised to undergo D-to-J_H rearrangement (Morshead et al., 2003), and in pre-B cells, activation of an Ig κ allele for V(D)J recombination is accompanied by increased methylation of H3 K4 (Goldmit et al., 2005). In contrast, dimethylation of histone H3 on lysine 9 (H3K9me2) is associated with silent chromatin and is correlated with inhibition of V(D)J recombination (Johnson et al., 2004; Osipovich et al., 2004).

RAG-1 and RAG-2 are 1040 and 527 amino acid residues long. Residues 384–1008 of RAG-1 constitute the core fragment, which contains the catalytic site for DNA cleavage (Fugmann et al., 2000b; Kim et al., 1999; Landree et al., 1999), mediates RSS binding (Akamatsu and Oettinger, 1998; Difilippantonio et al., 1996; Swanson and Desiderio, 1998), and contacts the coding flank (Eastman et al., 1999; Swanson and Desiderio, 1999). The core RAG-2 fragment, consisting of residues 1–387, extends interactions of RAG-1 with the RSS and is essential for helical distortion near the scissile bond; this distortion is a possible prerequisite for transesterification (Akamatsu and Oettinger, 1998; Difilippantonio et al., 1996; Hiom and Gellert, 1997; Swanson and Desiderio, 1998). Accordingly,

mutations that impair recombinase-mediated cleavage and joining have been identified in core RAG-2 (Qiu et al., 2001).

Residues 387–527 of RAG-2 are dispensable for DNA cleavage by the RAG proteins in vitro. Nonetheless, removal of this region reduces the efficiency of extrachromosomal recombination (Cuomo and Oettinger, 1994; Kirch et al., 1998; McMahan et al., 1997; Sadofsky et al., 1994; Sadofsky et al., 1993; Steen et al., 1999), increases production of hybrid joints (Sekiguchi et al., 2001), impedes endogenous V_H-to-DJ_H joining (Akamatsu et al., 2003; Kirch et al., 1998; Liang et al., 2002), and promotes aberrant recombination (Talukder et al., 2004). The mechanisms underlying these effects might be complex because the noncore region includes multiple functional domains.

Residues within RAG-2's noncore region, including a phosphorylation site for cyclin A-Cdk2 at Thr490 and the interval spanning amino acids 499–508, couple V(D)J recombination to the G1 cell cycle phase by supporting Skp1-Cul1-Skp2 (Skp2-SCF)-dependent polyubiquitylation and destruction of RAG-2 at the G1-S transition (Jiang et al., 2005; Lee and Desiderio, 1999; Li et al., 1996). Periodic destruction of RAG-2 might protect against aberrant joining of RAG-induced DNA breaks generated outside of G1.

More recently, a noncanonical plant homeodomain (PHD) finger has been identified within residues 419–481 of the noncore region of RAG-2 (Callebaut and Mornon, 1998; Elkin et al., 2005). The canonical PHD finger occurs in a number of proteins associated with epigenetic regulation of gene expression (Bienz, 2006; Mellor, 2006) and specifically binds H3K4me2 or histone H3, trimethylated at lysine 4 (H3K4me3) (Li et al., 2006; Pena et al., 2006; Shi et al., 2006; Wysocka et al., 2006). Indeed, a carboxy-terminal RAG-2 fragment, spanning the noncanonical PHD finger, was reported to interact with histones (West et al., 2005). Moreover, mutations within this domain are associated with immunodeficiency disorders (Gomez et al., 2000; Noordzij et al., 2002; Schwarz et al., 1996; Villa et al., 2001).

In this communication, we now show that the PHD finger of RAG-2 specifically bound H3K4me2 or H3K4me3 and that this interaction mediated association of RAG-2 with rearranging gene segments. We demonstrated that in the context of full-length RAG-2, the PHD finger was critical for efficient rearrangement of extrachromosomal V(D)J recombination substrates and of endogenous immunoglobulin gene segments.

RESULTS

Binding of RAG-2 to Histone H3 Dimethylated or Trimethylated at Lysine 4

To determine whether intact RAG-2 could bind specifically to H3K4me3, we first assayed binding to synthetic peptides derived from histone H3. A lysate of 293T cells expressing wild-type, murine RAG-2 was incubated with beads bearing an unmodified peptide corresponding to residues 1–21 of histone H3 or with beads bearing a similar peptide that was trimethylated at lysine 4. RAG-2 was precipitated by beads attached to the H3K4me3 peptide but not by beads bound to the unmodified peptide (Figure 1A, compare lanes 2 and 4). To probe the specificity of this interaction, we assayed a series of histone H3-derived peptides for binding. RAG-2 was precipitated by beads bearing the H3K4me3 peptide and was precipitated less efficiently by the corresponding H3K4me2 peptide (Figure 1B, lanes 4 and 5). No association was detected between RAG-2 and the unmodified histone H3 peptide (Figure 1B, lane 2), histone H3 monomethylated at lysine 4 (H3K4me1) (Figure 1B, lane 3), or the corresponding peptide trimethylated at lysine 9 (Figure 1B, lane 6). RAG-2 also failed to bind to a peptide spanning residues 21–44 of histone H3, which was trimethylated at lysine 27 (Figure 1B, lane 7).

These results were consistent with specific binding of RAG-2 to H3K4me2 and H3K4me3, with stronger binding to the trimethylated derivative. This was confirmed with surface plasmon resonance (SPR). Biotinylated peptides corresponding to H3K4me1, H3K4me2, and H3K4me3 were affixed to streptavidin-coated sensor chips at similar densities and assayed for binding to a glutathione-S transferase (GST) fusion protein (GST-RAG-2_{PHD}) containing residues 388-494 of RAG-2, which span the noncanonical PHD finger, and the linker that connects the PHD finger to the RAG-2 core (Callebaut and Mornon, 1998). SPR response curves were consistent with affinities of the RAG-2 noncore region for H3 peptides in the order H3K4me3 > H3K4me2 > > H3K4me1 (Figure 1C).

We proceeded to test the dependence of binding of GST-RAG-2_{PHD} to intact histone H3. In budding yeast, deletion of *SET1* abolishes methylation of histone H3 on lysine 4 (Briggs et al., 2001). Histones were isolated by acid extraction from wild-type and *set1* null yeast strains and assayed for binding to GST-RAG-2_{PHD}. Histone H3 from wild-type yeast was efficiently precipitated by GST-RAG-2_{PHD}; binding to H3 from cells lacking the Set1 H3K4 methyltransferase was greatly reduced (Figure 1D, compare lanes 2 and 4). Taken together, these results indicate that methylation of histone H3 at lysine 4 supports binding to RAG-2 in vitro.

Binding to Dimethyl and Trimethyl Lysine 4 of Histone H3 Mediated by the Noncanonical PHD Finger

To confirm that RAG-2 binding to H3K4me2 and H3K4me3 is mediated by the noncanonical PHD finger, we tested a series of RAG-2 truncation mutants (Figure S1 in the Supplemental Data available online) in the peptide precipitation assay. GST fusion proteins were expressed in bacteria, purified, and used in binding assays at similar concentrations (Figure 2A, left). As shown above, the GST-RAG-2_{PHD} fusion bound the H3K4me3 peptide, whereas a GST fusion to the RAG-2 core (residues 1–387) did not (Figure 2A, right, lanes 2 and 3). Sequence comparison reveals that residues corresponding to W453 and C478 of RAG-2 are highly conserved among PHD fingers (Ruthenburg et al., 2007). In crystal structures

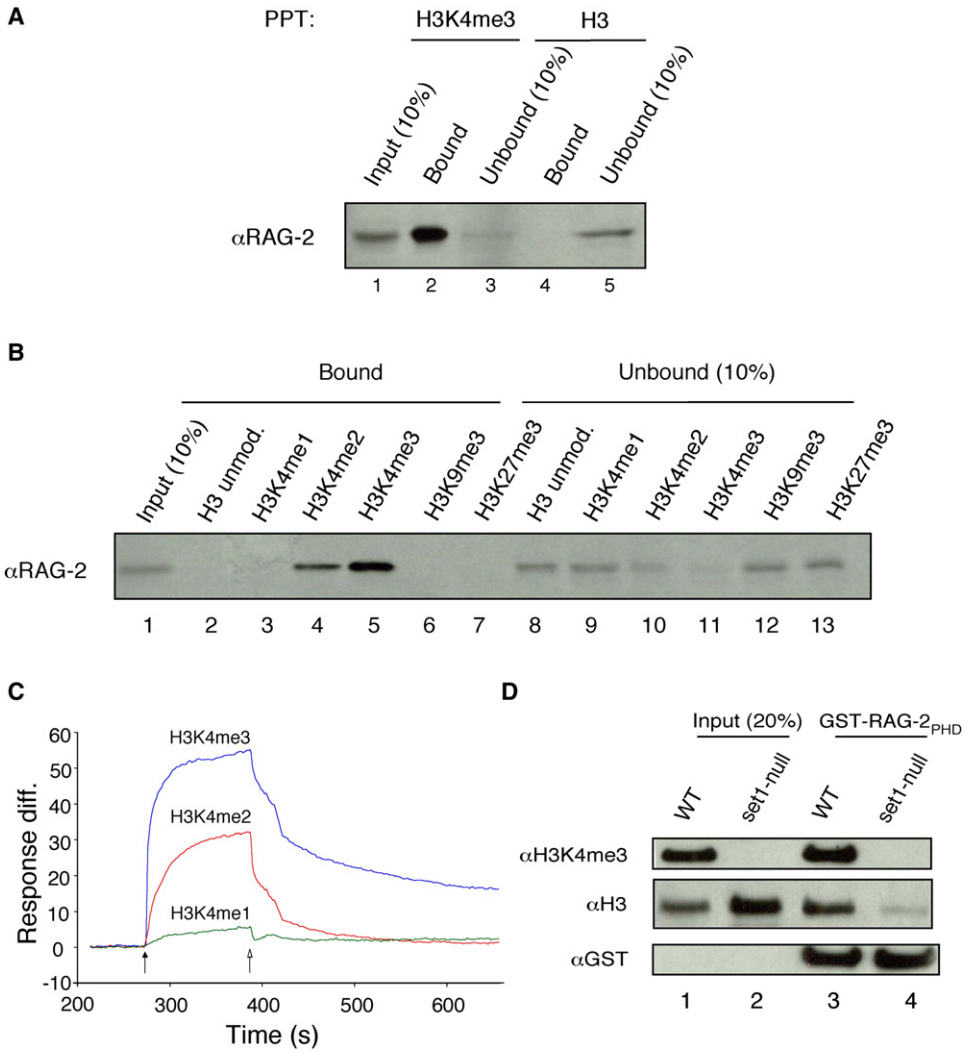

Figure 1. Specific Binding of RAG-2 to Histone H3 Containing Dimethylated or Trimethylated Lysine 4

(A) Whole-cell lysates of 293T cells expressing full-length, wild-type RAG-2 were incubated with streptavidin bead-bound, biotinylated peptides corresponding to residues 1–21 of histone H3. Peptides were unmodified (H3) or trimethylated at lysine 4 (H3K4me3). Bead-bound protein was fractionated by SDS-PAGE, and RAG-2 was detected by immunoblotting. Portions (10%) of the lysate (Input) or unbound protein were assayed in parallel.

(B) Lysates of 293T cells expressing wild-type RAG-2 were incubated with unmodified or modified histone H3 peptides, affixed to beads. Modifications are indicated above. All peptides correspond to residues 1–21 of histone H3 except for the H3K27me3 peptide, which corresponds to residues 21–44. Bound protein was fractionated as in (A), and RAG-2 was detected by immunobloting. Portions (10%) of the lysate (Input) or unbound protein were assayed in parallel.

(C) Surface-plasmon-resonance binding curves for GST-RAG-2_{PHD} association with histone H3 peptides as a function of lysine 4 methylation. Biotin-tagged H3 peptides (residues 1–21) were immobilized at 0.06 ng each on flow cells of a sensor chip. GST-RAG-2_{PHD} (5 μM) was injected (solid arrow) for 2 min; this was followed by injection of buffer alone (open arrow). Binding curves were deduced after subtracting the response of the reference surface.

(D) Methylation at lysine 4 is required for binding of the RAG-2 PHD finger to intact histone H3 in vitro. GST-RAG-2_{PHD} was immobilized on glutathione-coated beads and incubated with histones isolated from wild-type or *set1* null *S. cerevisiae*. Histone H3 trimethylated at lysine 4, bulk H3, and GST were detected in input and bound fractions by immunoblotting.

of PHD domains from ING2 (Pena et al., 2006) and NURF (Li et al., 2006), a residue corresponding to RAG-2 W453 recognizes the trimethylammonium group of H3K4me3, whereas a residue corresponding to RAG-2 C478 coordinates Zn^{2+}. Moreover, mutations at the homologous positions are associated with immunodeficiency syndromes in humans (Gomez et al., 2000; Noordzij et al., 2002; Schwarz et al., 1996; Villa et al., 2001). We introduced single- and double-alanine substitutions at W453 and C478 of the GST-RAG-2_{PHD} fusion. The W453A mutation, but not the C478A mutation, abolished binding to the H3K4me3 peptide (Figure 2A, right, lanes 4 and 5); the W453A, C478A double mutant, like the W453A single mutant, failed to bind. These data are consistent with a direct

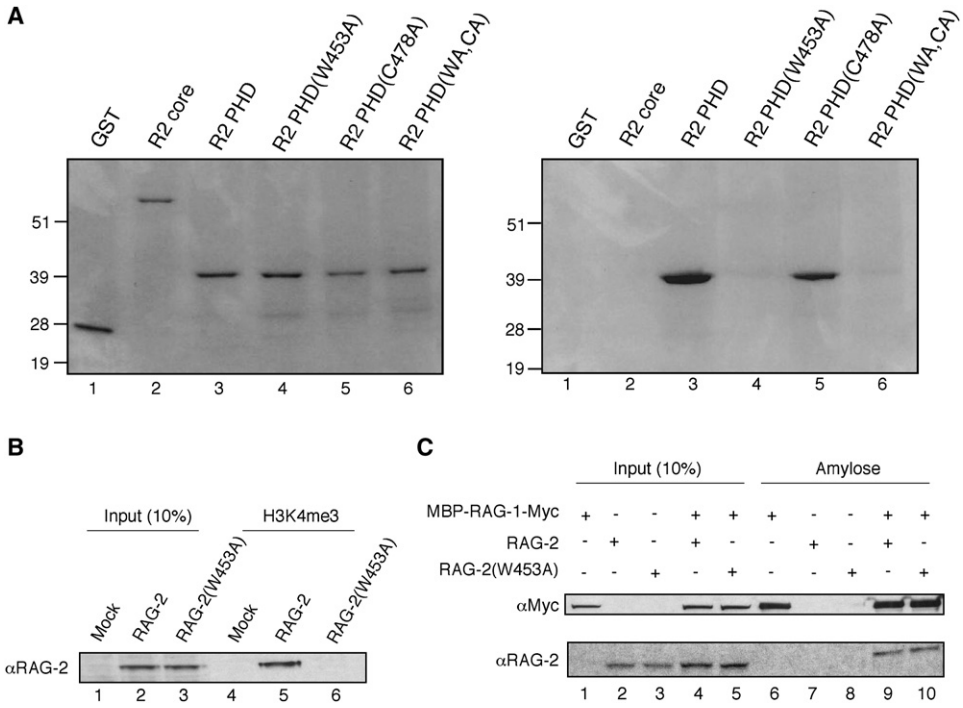

Figure 2. Binding of RAG-2 to Tri-Me H3K4 Is Mediated by the PHD Finger

(A) The RAG-2 PHD finger mediates direct binding to tri-Me H3K4. Proteins were expressed in *E.coli*, purified on glutathione agarose, and assayed for binding to biotinylated H3K4me3 peptide immobilized on streptavidin-linked beads. Bound protein (right) or 10% input protein (left) was fractionated by SDS-PAGE and detected by Coomassie blue.

(B) Lysates of mock transfected 293T cells or cells expressing full-length wild-type RAG-2 or RAG-2(W453A) were assayed for binding to the biotinylated H3K4me3 peptide as above (lanes 4–6). Bound protein was fractionated by SDS-PAGE and detected by immunoblotting for RAG-2. Input protein (10%) was analyzed in parallel (lanes 1–3).

(C) RAG-2(W453A) retains the ability to associate with RAG-1. Lysates of 293T cells coexpressing a c-*myc*-tagged MBP-RAG-1 fusion and RAG-2 or RAG-2(W453A) were adsorbed to amylose beads. Bound protein (lanes 6–10) was fractionated by SDS-PAGE and detected by immunoblotting for c-*myc* (top) or RAG-2 (bottom). Input protein (10%) was assayed in parallel (lanes 1–5).

interaction between the noncanonical PHD domain of RAG-2 and histone H3 trimethylated at lysine 4.

The effects of these mutations were also tested in the context of full-length RAG-2. Cells were transfected with wild-type RAG-2 or the RAG-2(W453A) mutant, and protein was precipitated from cell lysates by H3K4me3 peptide affixed to beads. The W453A mutation abolished binding to H3K4me3 peptide in this assay (Figure 2B, compare lanes 5 and 6). Importantly, the RAG-2(W453A) mutant retained the ability to associate with RAG-1 in transfected cells (Figure 2C), suggesting that this mutation does not disrupt the overall structure of the protein. Taken together, these observations indicate that the noncanonical PHD finger mediates binding of RAG-2 to hypermethylated lysine 4 of histone H3.

Association between Endogenous RAG-2 and Hypermethylated Histone H3 K4 from Primary Pro-B Cells

We next sought to detect an association between endogenous RAG-2 and histone H3 containing dimethylated or trimethylated lysine 4. In mice bearing a homozygous, targeted mutation of the IgM transmembrane region, RAG-expressing B cell progenitors accumulate at the pro-B-to-pre-B transition (Kitamura et al., 1991). Bone marrow cells were collected from 6-week-old, μM-deficient mice and incubated in the presence of IL-7 for 10 days so that a homogeneous culture of $CD43^{+}B220^{+}$ pro-B cells could be obtained. Protein was immunoprecipitated from cell lysates with an antibody specific for the di- and tri-Me K4 forms of histone H3. Precipitation of histone H3 bearing trimethylated K4 was monitored by immunoblotting (Figure 3, middle, lane 3). Endogenous RAG-2 coimmunoprecipitated with histone H3 bearing dimethyl and trimethyl K4 but was not detected in a control immunoprecipitate (Figure 3, top, lanes 2 and 3). We infer that a portion of RAG-2 is associated in vivo with histone H3 containing dimethylated or trimethylated lysine 4.

Binding of the RAG-2 PHD Finger to Chromatin in the IgH D-J_H-C Region Is Correlated with the Density of Trimethylated Histone H3 K4

Hypermethylated histone H3 K4 marks active chromatin domains and is associated with antigen-receptor-gene segments that are poised to undergo V(D)J recombination (Goldmit et al., 2005; Morshead et al., 2003). The ability of

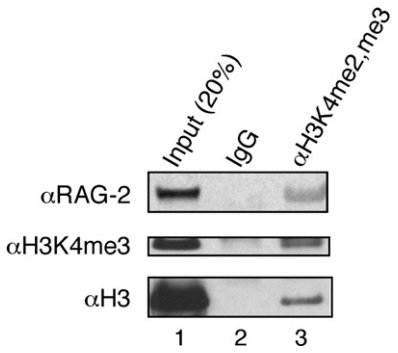

Figure 3. Pro-B Cell Lysates Contain RAG-2 Bound to Histone H3 Dimethylated or Trimethylated at Lysine 4

We cultured bone marrow cells in IL-7 for 10 days to generate $B220^{+}CD43^{+}$ pro-B cells. Protein was immunoprecipitated from pro-B cell lysates with an antibody specific for H3K4me2 and H3K4me3 or with an isotype control. Immunoprecipitates (lanes 2 and 3) were fractionated by SDS-PAGE and assayed by immunoblotting for RAG-2 (top), H3K4me3 (middle), and histone H3 (bottom). Input protein (20%) was assayed as a reference (lane 1).

the RAG-2 PHD finger to bind H3K4me2 and H3K4me3 suggested that such modifications might direct association of the PHD finger with chromatin in the context of an antigen-receptor locus. To determine whether the RAG-2 PHD domain recognized modified histones associated with recombinationally active antigen-receptor loci, we used a modified chromatin immunoprecipitation (ChIP) procedure. Sheared chromatin was precipitated with purified GST-RAG-2_{PHD} or GST-RAG-$2_{PHD(W453A)}$, and this was followed by quantitative PCR analysis with primers directed to specific regions of the germline IgH locus (Figure 4A). We chose a set of amplicons that showed distinct association patterns with H3K4me2 and H3K4me3, with the reasoning that such a set might also test the selectivity of the RAG-2 PHD domain for each modification. By using chromatin prepared from the RAG-2-deficient pro-B cell line 63-12 (Shinkai et al., 1992), we found that DFL16.1 and Cμ amplicons were enriched in anti-H3K4me2 immunoprecipitates compared to amplicons centered over J_H2 and J_H4 (Figure 4B, left). Conversely, J_H2 and J_H4 amplicons were enriched in anti-H3K4me3 immunoprecipitates (Figure 4B, middle). Cγ3 and *Actg1* (γ-actin) promoter primers served as negative and positive controls. In these experiments, we observed a striking correspondence between the pattern of amplicon enrichment in chromatin precipitated with GST-RAG-2_{PHD} and anti-H3K4me3 immunoprecipitation (Figure 4B, right). We observed no specific binding to the GST-RAG-$2_{PHD(W453A)}$ mutant. These results are consistent with a higher affinity of the RAG-2 PHD finger for trimethylated versus dimethylated histone H3 lysine 4 in the context of cellular chromatin and suggest that this modification might play a role in directing the recombinase to poised antigen-receptor loci. Taken together, our observations suggest that trimethylation of H3 K4 plays a dominant role in defining the specificity with which the RAG-2 PHD finger binds cellular chromatin.

Disruption of H3K4me3 Binding by the RAG-2 PHD Finger Impairs Recombination of Endogenous Ig Gene Segments and Extrachromosomal Substrates

Having found that the W453A mutation abolished binding of the RAG-2 PHD finger to H3K4me3 and to chromatin at the D-J_H-C locus, we wished to test the effect of this mutation on V(D)J recombination. Because rearrangements of endogenous antigen-receptor genes are subject to epigenetic constraints, we tested the ability of the RAG-

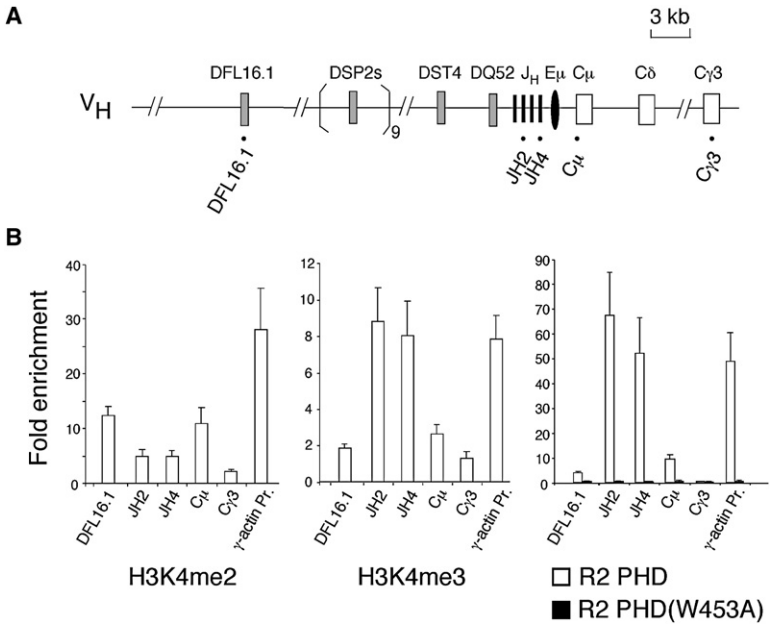

Figure 4. Binding of the RAG-2 PHD Finger to Chromatin in the IgH D-J_H-C Region Is Correlated with the Density of Trimethylated Histone H3K4

(A) Schematic representation of the IgH D-J_H-C region through the Cγ3 exons. Gray boxes represent D gene segments. Black vertical lines represent J_H gene segments; open boxes represent Cμ, Cδ, and Cγ3 exons. Black oval represents Eμ intronic enhancer. Black dots below indicate the approximate locations of primer pairs for the segments indicated.

(B) Precipitation of chromatin from the RAG-2-deficient 63-12 cell line with an H3K4me2 antibody (left), an H3K4me3 antibody (middle), wild-type GST-RAG-2_{PHD} (right, open bars), or the mutant GST-RAG-$2_{PHD(W453A)}$ (right, filled bars). DNA recovered from chromatin precipitates was analyzed by quantitative real-time PCR (qRT-PCR). The fold enrichment of each amplified DNA fragment, indicated below as in (A), was determined relative to input DNA and then normalized to that of α-actin. The promoter region of *Actg1* (γ-actin) was amplified as a positive control. Error bars indicate SDs (n = 3).

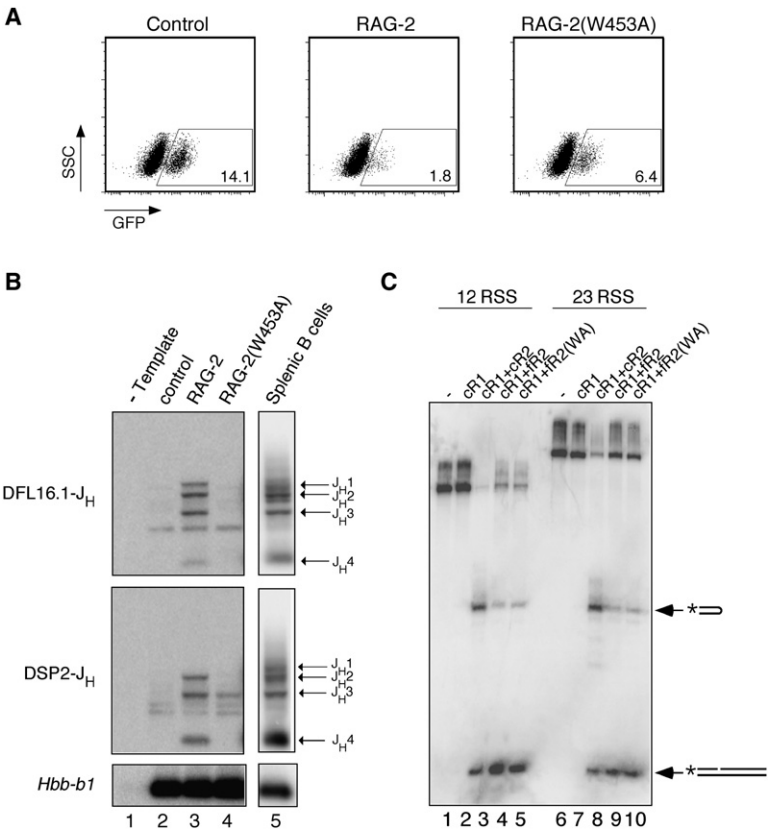

Figure 5. A Mutation Abolishing Binding of H3K4me3 by the RAG-2 PHD Finger Impairs Recombination of Endogenous-Immunoglobulin-Gene Segments in Pro-B Cells

(A) EGFP expression in infected cells. *Rag2*$^{-/-}$ R2FL63-12 pro-B cells were infected with control lentivirus or with a lentivirus expressing wild-type RAG-2 or RAG-2(W453A). Infection was confirmed by flow-cytometric detection of EGFP, which was encoded by all three lentiviruses, at 2 days after infection. Numbers indicate percentages of cells in gated areas. RAG-2 mRNA was assayed by qRT-PCR amplification of reverse transcripts from total RNA isolated 8 days after infection. The amounts of RAG-2 mRNA in cells infected with control, RAG-2, or RAG-2(W453A) lentiviruses, relative to γ-actin mRNA, were 0.00, 1.00, and 3.47, respectively.

(B) Detection of completed D-to-J_H rearrangements. D-to-J_H rearrangements were assayed 8 days after infection by PCR with genomic DNA from control- or RAG-2-infected pro-B cells as template as indicated above. Amplification was performed in the absence of template in parallel ("− Template"). Forward primers were specific for DFL16.1 (top panel) or DSP2 (middle panel); the reverse primer initiates synthesis 3′ of J_H4. Products were separated by gel electrophoresis and detected by hybridization to a radiolabeled, locus-specific probe that recognizes all J_H segments. Genomic DNA from C57BL/6 mouse spleen was used as a positive control for D-to-J_H rearrangements. The *Hbb-b1* locus (bottom panel) was amplified as a control for gel loading.

(C) RAG-2(W453A) retains catalytic activity in vitro. Core RAG-1 (cR1) and complexes of core RAG-1 with core RAG-2 (cR2), full-length RAG-2 (fR2), or full-length RAG-2(W453A) (fR2[WA]) were assayed in vitro for cleavage of 12-RSS (lanes 1–5) or 23-RSS (lanes 6–10) substrates; dashes represent that no protein added. Positions of nicked and hairpin products are indicated.

2(W453A) mutant to support endogenous IgH gene rearrangement. We expressed wild-type RAG-2 or RAG-2(W453A) in a RAG-2-deficient pro-B cell line by using a dual-expression lentiviral vector that produces GFP from a cassette residing downstream of an internal ribosomal entry site (Figure 5A). To assess recombination of the endogenous IgH locus in transduced pro-B cells, we harvested genomic DNA 8 days after infection and joining of DFL16.1 and DSP2 gene segments to J_H1–J_H4 was detected with the polymerase chain reaction (PCR) and Southern hybridization as described. Rearrangements of both D segment families were readily detected in cells transduced with wild-type RAG-2 (Figure 5B, lanes 3). In contrast, DFL16.1-to-J_H rearrangements were undetectable, and DSP2-to-J_H rearrangements were profoundly reduced in cells transduced with RAG-2(W453A) (Figure 5B, lanes 4). To eliminate the possibility that W453A renders RAG-2 catalytically inactive, we tested the ability of RAG-2(W453A) to support RSS-dependent DNA cleavage in vitro (Bergeron et al., 2006). In the presence of core RAG-1, equivalent amounts of wild-type RAG-2 and RAG-2(W453A) supported similar levels of nicking and transesterification on 12-RSS and 23-RSS substrates (Figure 5C). The correlation between loss of H3K4me2 and H3K4me3 binding and impaired rearrangement of endogenous Ig gene segments observed for the RAG-2(W453A) mutant is consistent with the interpretation that recognition of hypermethylated histone H3 K4 is required for the support of efficient V(D)J recombination by full-length RAG-2.

We also examined the effect of the W453A mutation on rearrangement of the extrachromosomal substrates pJH200 and pJH290, which report formation of signal joints and coding joints, respectively (Hesse et al., 1987; Lieber et al., 1988), in NIH 3T3 cells. In cells of murine origin, these plasmids replicate and are organized into chromatin (Baumann et al., 2003). Moreover, by using ChIP we found that in NIH 3T3 cells, pJH200 is associated with histone H3 and H3K4me3, whereas a nonreplicative plasmid, pcDNA3, showed no such association (Figure 6A). The RAG-2(W453A) mutation reduced the frequency of signal (pJH200, $p < 0.03$) and coding (pJH290, $p < 0.04$) joint formation to 3% of the wild-type (Figure 6B, upper panel). This is in agreement with the effect of a W453R mutation on rearrangement of extrachromosomal substrates (Elkin et al., 2005). In contrast, complete removal of the RAG-2 noncore region had a much less debilitating effect, reducing rearrangement

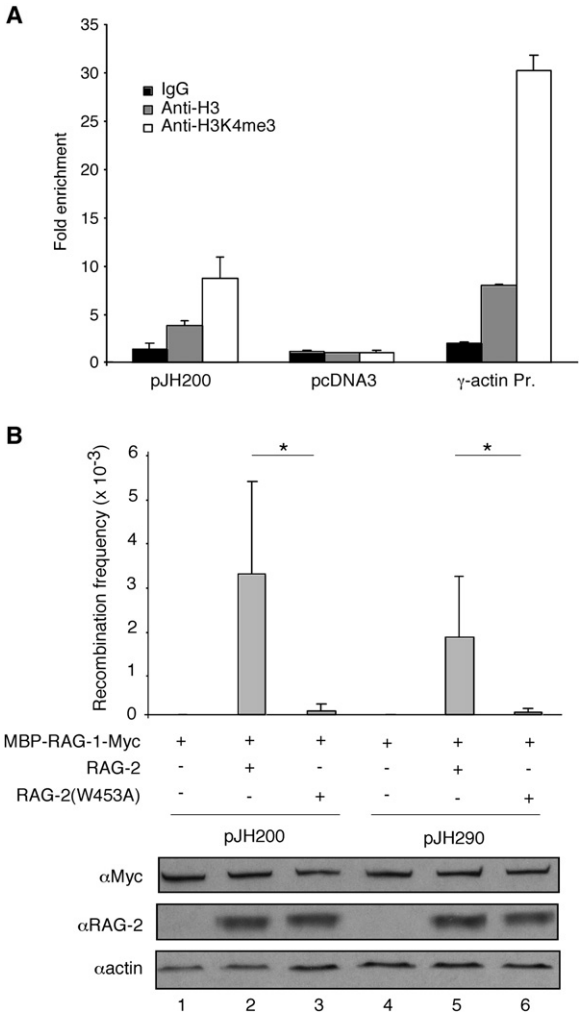

Figure 6. A Mutation Abolishing Binding of H3K4me3 by the RAG-2 PHD Finger Impairs Recombination of Extrachromosomal Substrates

(A) Association of pJH200 with histone H3 and H3K4me3 in NIH 3T3 cells. Chromatin was precipitated from transfected cells with control IgG (filled bar), anti-H3 (shaded bar), or anti-H3K4me3 (open bar) and detected by qRT-PCR. Plasmid pJH200 was detected by amplification across the 12-spacer RSS; the cotransfected, nonreplicative plasmid pcDNA3 was assayed by amplification of *neo*. The endogenous promoter region of *Actg1* (γ-actin) was amplified as a positive control. The fold enrichment of each amplified DNA fragment was determined relative to input DNA and then normalized to that of the *f1* origin region of pcDNA3. Error bars indicate SDs (n = 2).

(B) Plasmids encoding a c-*myc*-tagged MBP-RAG-1 fusion and RAG-2 or RAG-2(W453A) were transiently cotransfected with the recombination substrate pJH200 or pJH290 into NIH 3T3 cells. In control experiments, plasmid pcDNA3 was transfected instead of the RAG-2-encoding plasmid. Plasmids were introduced in the combinations indicated. At 48 hr after transfection, plasmids were recovered and transfected into *E. coli*; transfectants were scored for resistance to ampicillin (Amp^r) or ampicillin and chloramphenicol (Amp^r + Cam^r). Recombination frequency (mean ± SD, n = 2) was calculated as the ratio of double to single resistant colonies (Amp^r + Cam^r/Amp^r). *$p < 0.04$, two-tailed t test. Cell lysates were analyzed by immunoblotting for RAG-1 (c-*myc*), RAG-2, or actin as indicated.

of pJH200 and pJH290 to 26% and 48% of the wild-type, respectively (data not shown), consistent with published results (Cuomo and Oettinger, 1994; Sadofsky et al., 1994). Possible reasons for the difference in activity between core RAG-2 and RAG-2(W453A) are discussed in the next section. Moreover, the accumulation of RAG-2(W453A) (Figure 6B, bottom panel) and its association with RAG-1 were similar to wild-type RAG-2. Together with the ability of RAG-2(W453A) to catalyze DNA cleavage in vitro, these observations suggest that the effect of the W453A mutation on recombination is more directly related to its debilitating effect on binding to hypermethylated histone H3 lysine 4.

DISCUSSION

Trimethylation of histone H3 at lysine 4 is an evolutionarily conserved posttranslational modification associated with transcription start sites in yeast and metazoans (Sims and Reinberg, 2006). H3K4me3 is specifically bound by a methyl-lysine-binding domain, the PHD finger, which has been found in subunits of a number of multiprotein complexes that execute the posttranslational modification of histones. These include the ACF1 component of the ATP-dependent chromatin assembly factor (ACF) chromatin remodeling complex (Eberharter et al., 2004), the histone acetyltransferases CBP and p300 (Kalkhoven et al., 2002), ING1 and ING2, which are associated with Sin3-HDAC1,2 histone deacetylase complexes (Shi et al., 2006), and ING3, which is a part of the NuA4 histone acetyltransferase complex (Doyon et al., 2006). In these instances, the PHD finger might promote region-specific chromatin modifications through recognition of H3K4me3 (Ruthenburg et al., 2007).

We have shown here that a noncanonical PHD finger within RAG-2 was also capable of specific binding to H3K4me3 and have provided evidence that this interaction supports the specific association of RAG-2 with subregions within the IgH locus in chromatin from cells poised to undergo D-to-J_H rearrangement. Our results indicate that the patterns of H3K4me2 and H3K4me3 over the IgH locus in pro-B cells are distinct and suggest that the density of H3K4me3 determines binding of the RAG-2 PHD finger to chromatin at the IgH D-J_H-C locus. Moreover, a point mutation that abolished specific binding to hypermethylated H3 K4 also profoundly impaired recombination of endogenous gene segments in pro-B cells. These results shed light on the contributions of the RAG-2 noncore region to V(D)J recombination and raise several questions concerning the function of the RAG-2 PHD finger. We will consider these issues below in the context of locus specificity.

At the level of unchromatinized DNA, the V(D)J recombinase is targeted to antigen-receptor-gene segments by means of specific interactions with flanking recombination signal sequences (RSS), and this recognition does not require the noncore regions of RAG-1 or RAG-2. Not all RSSs support recombination with the same efficiency, and sequence variation among RSSs can affect gene-segment

usage (Feeney, 2000). Nonetheless, these differences do not account for the tightly regulated locus specificity that V(D)J recombination exhibits with respect to lymphoid lineage and developmental stage. Rather, ordered rearrangement of antigen-receptor-gene segments is associated with the imposition or relief of epigenetic marks including changes in histone methylation. As in the case of transcriptional activation, different methylation states might have distinct functional consequences. In particular, the relative distributions of H3K4me2 and H3K4me3 at antigen-receptor loci as a function of recombination activity have not yet been examined systematically.

Several amino acid substitutions identified as causes of severe combined immune deficiency (SCID) or Omenn syndrome in humans (Gomez et al., 2000; Noordzij et al., 2002; Schwarz et al., 1996; Villa et al., 1998; Villa et al., 2001), including W453R, N474S, C478F, and H481P, reside within the RAG-2 PHD finger. One of these mutations, W453R, unambiguously impairs signal- and coding-joint formation in an extrachromosomal assay for recombination (Elkin et al., 2005). We have shown here that an alanine substitition at W453 eliminated binding of the RAG-2 PHD finger to hypermethylated H3 K4 and correspondingly to chromatin at the IgH D-J_H-C locus in pro-B cells. The W453A mutation, like the W453R mutation, impaired recombination of extrachromosomal substrates. Importantly, we observed a similar impairment of D-to-J_H joining at endogenous loci in pro-B cells, despite the fact that RAG-2(W453A) retained catalytic function in vitro and that accumulation of this mutant and its association with RAG-1 were similar to wild-type RAG-2's accumulation and its association with RAG-1. The susceptibility of extrachromosomal substrates to the debilitating effect of the W453A mutation might be explained by the fact that these substrates replicate in permissive cells, in which they are associated with chromatin (Baumann et al., 2003) and more specifically with histone H3 bearing trimethylated lysine 4. Thus, we favor the interpretation that impaired recombination in the context of full-length RAG-2 is due to loss of H3K4me3 binding.

This result seemed paradoxical for several reasons. First, complete removal of the RAG-2 noncore region only reduced recombination to between 30% and 50% of the wild-type in extrachromosomal assays (Cuomo and Oettinger, 1994; Sadofsky et al., 1994), as we confirmed. Second, although a knockin mutation that removes the RAG-2 noncore region is associated with impaired rearrangement of V_H gene segments, there was little or no effect on D-to-J_H joining (Akamatsu et al., 2003; Liang et al., 2002). Third, enforced expression of core RAG proteins in R2FL63-12 pro-B cells rescues the defect in D-to-J_H joining (Kirch et al., 1998), in contrast to the impaired ability of the full-length RAG-2(W453A) mutant to support recombination.

An economical way to reconcile the behavior of core RAG-2 and the RAG-2(W453A) mutant is to propose that the noncore region contains an inhibitory domain whose function is relieved by binding of the PHD finger to H3K4me3. In this model, impairment of binding by the PHD finger, such that occurs in the RAG-2(W453A) mutant, would result in constitutive inhibition of RAG-2 activity at chromatinized substrates in vivo. Conversely, complete removal of the noncore region, aside from its effects on programmed degradation, would produce a constitutively active form of RAG-2 in which activity is uncoupled from interaction with H3K4me3. In this model, hypermethylated H3 K4 would not simply represent a docking site for the RAG proteins but would play an active role through allosteric activation of recombinase function.

The ability of the RAG-2 PHD finger to bind hypermethylated histone H3 K4 in general is in agreement with its ability to interact with chromatin at loci other than antigen-receptor genes, such as *Actg1*. Clearly, modes of regulation other than recognition of hypermethylated histone H3 K4 must determine the locus specificity of recombinase activity. One possibility is that RSS recognition by the RAG heterodimer ensures productive binding only to antigen-receptor-gene segments. Another possibility, not exclusive of the first, is that specificity is determined by chromatin modifications other than or in addition to hypermethylation on H3 K4. Regardless of such additional specifiers, the recruitment of the RAG complex to the vicinity of poised antigen-receptor loci by hypermethylated H3 K4 might itself enhance recombination through allosteric effects on activity against chromatinized substrates, as argued above, but also by increasing the local availability of the recombinase.

EXPERIMENTAL PROCEDURES

Antibodies

Antibodies against the following were obtained commercially: histone H3 (Abcam, Ab1791); H3K4me2 and H3K4me3 (Abcam, Ab6000); H3K4me3 (Abcam, Ab8580); GST (Santa Cruz, sc-459); actin (Santa Cruz, sc-8432); and c-*myc* (Upstate Biotechnology, CBL-430). RAG-2 Ab435 has been described (Lin and Desiderio, 1993).

Expression Constructs and Site-Directed Mutagenesis

Plasmid pcRAG-2 (Ross et al., 2003), encoding full-length RAG-2, and plasmids encoding maltose-binding protein (MBP) fusions to full-length RAG-1, core RAG-1, or full-length RAG-2, tagged at the c-termini with a Myc epitope and polyhistidine (McBlane et al., 1995; Jiang et al., 2004), have been described. Mutagenesis and lentiviral constructions are described in the Supplemental Data.

Cell Culture

Cell-culture conditions are provided in the Supplemental Data.

GST Fusion Proteins

DNA fragments encoding core RAG-2 (residues 1–387) or the RAG-2 PHD finger (residues 388 - 494) were amplified by PCR from wild-type or mutant RAG-2 constructs and subcloned in frame into pGEX-5X-1 (Amersham) between the *Eco*R1 and *Not*I restriction sites. All constructs were confirmed by nucleotide sequencing. GST fusion proteins were expressed in *E. coli* (Star BL21, Invitrogen) and purified by glutathione-agarose affinity chromatography (Jiang et al., 2005).

Protein-Binding Assays

Biotinylated peptides derived from histone H3 were purchased from Upstate Biotechnology. Whole-cell lysates were prepared from 293T

cells by lysis in a buffer containing 50 mM Tris (pH 7.5), 300 mM NaCl, 1 mM PMSF, 1% NP-40, 1% deoxycholic acid, 0.1% SDS, and a cocktail of protease inhibitors (Roche). After centrifugation at 16,000 g for 15 min, the supernatant was diluted 10-fold in binding buffer (50 mM Tris [pH 7.5], 300 mM NaCl, 1 mM PMSF, and protease inhibitors). The diluted supernatant, containing 300 μg protein, was precleared with 50 μl of a 50% streptavidin agarose slurry (Novagen) and incubated with 5 μg peptide prebound to streptavidin agarose for 4 hr at 4°C. Beads were washed three times with binding buffer supplemented with 0.1% NP-40.

Yeast histones were prepared by acid extraction (Edmondson et al., 1996) from wild-type or *set1* null strains of *S. cerevesiae* (Park et al., 2002), provided by Dr. Jef D. Boeke. For binding assays to yeast histone H3, 15 μg of acid extracted yeast histones were incubated for 4 hr at 4°C in binding buffer supplemented with 0.1% NP-40 with 2.5 μg wild-type or mutant GST-tagged RAG-2_{PHD} protein that had been preadsorbed to glutathione-agarose. Beads were washed three times with binding buffer supplemented with 0.1% NP-40.

To detect association of wild-type and mutant RAG-2 with MBP-tagged RAG-1-Myc-His, we lysed 293T cells transfected with the corresponding expression constructs and isolated complexes by amylose affinity chromatography as described (Jiang et al., 2004).

Surface Plasmon Resonance

Surface-plasmon-resonance binding assays were performed on a BIAcore 3000 biosensor with immobilized H3 peptides, monomethylated, dimethylated, or trimethylated on lysine 4 (0.06 ng each). GST-tagged RAG-2_{PHD} protein (5 μM) was injected for 2 min at a flow rate of 10 μl/min; this was followed by injection of running buffer alone for 5 min. A detailed description is provided in the Supplemental Data.

Immunoprecipitation

Primary pro-B cells were obtained from μM-deficient, C57BL/6 mice (Jackson Laboratory Strain 002288). Procedures conform to the regulatory standards of the Johns Hopkins Animal Care and Use Committee, which approved the protocols. Cells were lysed at 4°C for 1 hr in a buffer containing 50 mM Tris (pH 7.5), 300 mM NaCl, 1 mM PMSF, 1% NP-40, 1% deoxycholic acid, 0.1% SDS, and a cocktail of protease inhibitors (Roche). After centrifugation for 15 min at 16,000 g the supernatant, containing about 500 μg protein, was diluted 10-fold in immunoprecipitation buffer (50 mM Tris [pH 7.5], 300 mM NaCl, 1 mM PMSF, and protease inhibitors) and precleared with 50 μl protein A/G agarose (50% slurry). The precleared supernatant was incubated for 4 hr at 4°C with 10 μg antibody. Protein A/G agarose beads (50 μl of a 50% slurry) were added and incubation, with rocking, was continued for another 2 hr at 4°C. After centrifugation for 1 min at 200 g, beads were collected and washed three times for 10 min each with 1 ml immunoprecipitation buffer containing 0.1% NP-40. Pellets were suspended in SDS loading buffer, heated to 95°C for 5 min and fractionated by SDS-PAGE on a 4%–12% Bis-Tris gel in NuPAGE MES running buffer (Invitrogen).

Chromatin-Precipitation Assays

Assays were performed with a Chromatin Immunoprecipitation Assay Kit (Upstate, 17-295) according to the manufacturer's instructions. Between 4 and 5 × 10^6 63-12 pro-B cells were used for each assay. For precipitation by the RAG-2 PHD domain, 10 μg of purified GST fusion protein and 50 μl glutathione agarose (75% slurry) were used in each assay. For chromatin immunoprecipitation (ChIP) experiments, 10 μg antibody and 60 μl protein A/G agarose (50% slurry) were used in each assay. For ChIP assays of plasmid DNA, 10 μg pcDNA3 and 4 μg of pJH200 were cotransfected into NIH 3T3 cells. At 40–48 hr after transfection, ChIP assays were performed as described above with 1 × 10^6 cells for each assay. Recovered DNA was amplified by quantitative real-time PCR with a SYBR Green PCR Master Mix (Bio-Rad), with a Bio-Rad iCycler iQ.

Primer sequences are given in the Supplemental Data.

In Vitro DNA Cleavage Assays

RAG fusion proteins were purified by amylose affinity chromatography as described (Bergeron et al., 2006). DNA cleavage reactions were performed in Mg^{2+} with the 12-RSS substrate DAR39/DAR40 or the 23-RSS substrate DAR61/DAR62 as described (Bergeron et al., 2006), except that core RAG-1 and full-length RAG-2 or RAG-2(W453A) were added at 400 ng each. Reactions were carried out at 37°C for 30 min.

Extrachromosomal Assays for V(D)J Recombination

Assays were performed as described (Hesse et al., 1987) with slight modification. In brief, plasmids (10 μg) encoding MBP-RAG-1-Myc-His and RAG-2 or RAG-2(W453A) were cotransfected with 4 μg pJH200 or pJH290 into NIH 3T3 cells, and plasmid DNA was isolated 40–48 hr after transfection. *E. coli* DH5α was transformed with 1 μl (approximately 40 ng) plasmid DNA, and 1.7% of each transformation mix was plated on LB agar containing 50 μg/ml ampicillin; the remainder was plated on LB agar containing 50 μg/ml ampicillin and 12.5 μg/ml chloramphenicol. Plates were scored after 14–16 hr at 37°C.

Retroviral Transduction

Lentiviral particles coexpressing EGFP and RAG-2 or RAG-2(W453A) were generated as described (Stewart et al., 2003). In brief, 293T cells were seeded 24 hr before transfection at 2 × 10^6 cells per 10 cm plate. The empty pWPI vector, pWPI-RAG-2, or pWPI-RAG-2(W453A) were cotransfected with the helper plasmids pΔ8.2R and pVSVG with FuGene 6 (Roche). Medium was replaced 24 hr after transfection, and supernatant was collected at 48 and 72 hr after transfection. Virus was concentrated by ultracentrifugation over a cushion of 20% sucrose in a SW32.1Ti rotor for 2 hr at 25,000 rpm and 20°C. *Rag2*$^{-/-}$ R2FL63-12 pro-B cells were infected with freshly prepared control, RAG-2-expressing, or RAG-2(W453A)-expressing lentivirus by spin inoculation in the presence of 10 μg/ml polybrene. Infection was confirmed by flow-cytometric detection of EGFP at 2 days after infection. Genomic DNA and total RNA were isolated from infected cells at 8 days after infection (DNeasy and RNeasy, QIAGEN).

Assay for Endogenous D-to-J_H Recombination

A PCR-based assay was used for detection of endogenous D-to-J_H recombination. Genomic DNA from transduced cells (90 ng) was amplified with forward primers specific for DFL16.1 (5′-ACACCT GCAAAACCAGAGACCATA-3′) or DSP2 (5′-ATGGCCCCTGACACT CTGCACTGC T-3′) and with a reverse primer that initiates synthesis from a site 3′ of J_H4 (5′-AAAGACCTGGAGAGGCCATTCTTACC-3′). Genomic DNA from C57BL/6 mouse spleen was used as a positive control template. Amplification was performed with the following protocol: 1 cycle at 95°C for 5 min; 33 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 3 min; 1 cycle at 72°C for 7 min. Products were detected by Southern hybridization to a degenerate oligonucleotide probe that recognizes all J_H segments (5′-CTYACCTGMRGAG ACDGTGAS-3′). The genomic DNA samples were normalized for quality and quantity by PCR amplification of the mouse *Hbb-b1* locus with primers 5′-GCCTTGCCTGTTCCTGCTC-3′ and 5′-CAGACCATA AACTGTATTTTTCTTATT-3′; this was followed by Southern hybridization to an *Hbb-b1*-specific probe (5′-GTGCATCTTGACTAGTTCCCA CACC-3′).

Supplemental Data

Additional Experimental Procedures, one figure, and one table are available at http://www.immunity.com/cgi/content/full/27/4/561/DC1/.

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